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14. ABSTRACT

Over the past year, we have generated potentially tumor-reactive T cell clones from a number of patient samples. We screened BC patients' CD8+ T cells against autologous tumor cells. We have identified TCR sequences in CD8 T cell clones which downstream analysis of antigen specificity will be assessed by the Denver team. The identification of these T cells will lead to downstream antigen discovery.

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Breast cancer, immunotherapy, vaccine, antigens

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INTRODUCTION

The immune response offers exquisite specificity and the potential to target tumor cells without harming normal cells. Inducing an effective immune response via therapeutic vaccines for cancer had been a promising but elusive goal for years. For breast cancer (BC), vaccine efforts have largely focused on eliciting immune responses to HER2. While HER2 is generally assumed to be a good antigen in HER2-overexpressing tumors, HER2-specific T cells exist at very low levels (less than 0.1%) in peripheral blood of such patients [1]. Hierarchy of the T cell repertoire and negative selection can shape immune responses in ways not readily predictable from protein expression levels alone. Thus, targeting a single antigen such as HER2 in breast cancer is likely to be insufficient - instead we need a repertoire of multiple immunologically validated T cell antigens present in breast cancers that can be deployed in a patient-specific manner. Research has focused on stimulating T cells using many pathways including the T cell antigen receptor (TCR), via co-stimulatory pathways, and manipulating the tumor environment. To optimally activate pre-existing anti-tumor T cells in BC patients, the antigens to which these T cells target must be determined. It is now recognized that invasive ductal carcinoma of the breast is a heterogeneous disease consisting of several major molecularly defined subtypes, including Luminal A, Luminal B, HER2+, and Basal (also known as 'triple-negative', and includes the 'claudin-low' subset). These subtypes have distinct clinical, genomic and proteomic features, and it is becoming clear that there are differences between BC subtype and response to specific therapeutic agent. These results, combined with the differences in gene expression that define the distinct subtypes, make it likely that each BC subtype elicits immune responses via distinct sets of antigens, and may evade T cell-mediated killing by distinct mechanisms. Based on these newly discovered features of BC and the host immune response, this project seeks to develop a robust portfolio of immunologically validated antigens for the major BC subtypes, including those that target breast cancer stem cells, that can be used in a patient-specific manner for therapeutic vaccination, as well as to identify drugs that can synergize with these novel immunotherapies. The ultimate goal is to match these antigens and drugs to each patient's tumor subtype, thereby treating each patient with the most potent combinations and opening the door to personalized immunotherapy for breast cancer. This multi-ream project will use a number of novel immunological approaches to look for evidence of BC subtype specific tumor-reactive T cells within the tumor and/or tumor-draining lymph nodes (TDLNs) including isolating, expanding and cloning tumor-reactive T cells which will culminate in a robust portfolio of immunologically validated antigens for the major breast cancer subtypes, including those that target breast cancer stem cells. We seek to expand and enhance the function of these pre-existing anti-tumor T cells in patients by discovering their natural antigens, and identifying mimotopes that broadly activate them with even higher potency. Furthermore, we will enhance the efficacy of these T cells by identifying existing drugs that promote cancer cell apoptosis but have little or no negative effect on T cells. All of these antigens and agents can be matched to each patient's tumor subtype and other molecular characteristics, thereby opening the door to personalized immunotherapy.

BODY:

Our team at City of Hope (CoH) consists of 1 assistant research professor, 1 post-doctoral fellow, and 3 research associates. We work closely with our surgery, medical oncology, and pathology colleagues via an honest broker to obtain samples from the operating room to pathology and to my laboratory. In addition, we continually refine our protocols to maximize recovery of immune cells from tumor and lymph node specimens, and to optimize methods for analysis of fresh samples by flow cytometry. Below is a summary of our progress in relation to our proposed SOW tasks:

Identify immunologically validated antigens by determining antigens recognized by anti-tumor T cells from patients with major subtypes of breast cancer.

1. Generate reagents and identify conditions for experiments to follow: months 1-40, Lee, Slansky, and Spellman
2. Enroll 100 patients with all major breast cancer subtypes from the City of Hope Cancer Center (CoH): months 1-36, Lee
3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee
4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee
5. Generation and initial analysis of T cell clones: months 1-40, Lee
6. Determine antigens as subtype-specific, stem-specific, or shared (Aim 4a): months 12- 40, Lee, Slansky and Spellman
7. Identify antigens that target breast cancer stem cells (Aim 3b): months 12-40, Lee, Slansky and Spellman

Patient Enrollment and Sample Acquisition

Our progress this year has focused on tasks 1-5. In 2015 (as of October), we have acquired new 17 breast cancer patients' specimens consisting of peripheral blood, lymph node and/or tumor. From those 17 patients, we have sent out 26 samples to the Slansky/Kappler group for TCR sequencing. Patient characteristics and HLA-A2 and HLA-DR4 results are summarized in Table 1 and Table 2, respectively. All participants were without a history of any immune disorder prior to breast cancer diagnosis and had their surgical treatments at City of Hope (CoH). Through an honest broker, written informed consent had been obtained from all participants according to CoH and HIPAA regulations using a tissue banking protocol. Patient peripheral blood samples, breast tumor tissue, and/or tumor draining lymph node (TDLN: non-sentinel lymph node and/or sentinel lymph node) were collected and have been utilized for research purposes. As mentioned in our previous report, our approach to identifying reactive T cells is focused on CD8+ T cells. While we are still using CD4+ T cells for TCR repertoire analysis by the Denver team, we have decided it is more efficient and productive to focus our efforts on CD8+ T cells. This is primarily due to the fact that most of our protocols and tools (antibodies, identified peptides, and peptide libraries) are restricted to HLA-A2, which is only useful for CD8+ T cells.

Process of Patient Samples

<u>HLA typing</u>

Patients' peripheral blood mononuclear cells (PBMCs) DNA HLA were typed through the Histocompatiblity lab at CoH. CoH's Histocompatibility Laboratory is fully accredited by The American Society of Histocompatibility and Immunogenetics (ASHI), College of American Pathologists (CAP), and Clinical Laboratory Improvement Amendments (CLIA 88). They carry out the typing using the sequence-specific oligonucleotide probe (SSOP) method. The SSOP method allows the HLA lab to define the HLA type of our patient subjects to the allele level (so called '4 digits'). Initially we are requesting for the allele level typing of subjects for only HLA-A2 and HLA-DR, but information on other alleles is available at a later date if desired.

Identify and isolate anti-tumor T cells from TDLNs and tumor samples

<u>Sequencing of TCR of tumor reactive T cell clones</u>

At COH, we are sequencing the TCR receptor of potential tumor antigen reactive clones for each cancer patient. This information guides us to generate full length recombinant TCR proteins that will be used to screen against

the bacteriophage peptide library the Slansky/Kappler team has created to identify unknown antigens. Also, the alpha-beta TCR sequence from autologous tumor-reactive T cell clones will be cross referenced with the T cell repertoire data generated by Slansky/Kappler team, to evaluate the robustness of the high throughput T cell repertoire approach in identifying TCR sequence of tumor reactive T cells. In our previous report, we amplified and fused Vα and Vβ amplicons during the PCR reaction using a primer nesting technique. Since then, we have further improved our protocol. Currently, we amplify Vα and Vβ amplicons separately during the PCR reaction and enrich the product during a second PCR. Briefly, RNA is extracted from tumor reactive clones and converted into cDNA using oligo(dT) followed by amplification of TCR alpha and beta genes using a primer mixture for priming Vα and Vβ. A small aliquot from the first PCR reaction is used to perform the second PCR reaction using primers Vα+ Cα and Vβ+Cβ, as separate reactions, which spans the constant region of Cα and Cβ with Vα and Vβ, yielding two separate bands (V alpha and V beta) of the TCR. The amplified products are visualized by agarose gel electrophoresis (Figure 1). The expected bands are then excised and purified using a gel extraction kit and submitted for Sanger sequencing using T3 or T7 primers to the COH Integrative Genomics Core. The TCR nucleotide sequences are analyzed using IMGT/V-QUEST to determine the identity of the V alpha and V beta of the TCR.

Clones from patients BC81 and BC85 were sequenced in the last year. These clones were believed to be tumor reactive as defined by CD107 mobilization in the presences of autologous tumor cells. While we were not able to formally prove they are T cell clones that are HLA-A2 restricted, due to lack of sufficient autologous tumor cells, we believe there is enough evidence to pass them down for further analysis by our UC-Denver collaborators. This method described above was used to identify the V alpha and V beta of the TCR for patient BC85, clone 4 and it was also used to identify the TCRs of the most dominant clones in patient BC81. Out of the 15 clones that were sequenced for BC81, there was one repeating sequence that appeared 6 times (Table 3). This information helped minimize the amount of clones that had to be re-screened to confirm T-cell reactivity and also gave us the sequence for these potential tumor reactive T cell clones. Knowing the TCR sequence for BC85, clone 4 and the dominant sequences for patient BC81 will be used for further downstream analysis of antigen specificity by the Denver team.

Growth of Autologous Cancer Cells

Autologous cancer cells grown *in-vitro* are an ideal target source for identification of bona fide cancer reactive T cell clones. Our tumor cell culture procedures involve cutting tissues into small pieces and mincing, followed by digestion in 5 ml of media, Liberase-TM from Roche, and DNase I from Sigma at 37C for 45min. These single cell suspensions are then either cryopreserved for later use or cultured as follows. The tumors cells are seeded on matrigel coated plates in DMEMF12 advanced media containing 5% fetal bovine serum, 0.4µg/mL hydrocortisone, 5µg/mL insulin, 10ng/mL EGF, 100ng/ml cholera toxin, and 1% pen/strep.

Generation and analysis of T cell clones

Generation of Tumor Reactive T cells

As described in previous reports, we have optimized conditions for the identification of tumor reactive T cells. Our two primary strategies have been to use mDCs pulsed with breast cancer cell line lysate and to use autologous tumor cells as targets.

In the former, we use 5-7 day generated autologous patient mDCs cultured overnight with 200ug/ml of breast cancer line lysate (prepared by the Spellman group) and then matured overnight with a monocyte maturation cocktail (TNFα, IL-1β, PDE2, IL-6). In the latter, we use cultured autologous tumor cells or cryopreserved tumor cells. Tumor cells are treated overnight with 750 IU/ml of IFNα to upregulate MHC Class I expression, which is known to be down-regulated on tumor cells. Following co-culture of patient derived T cells with either mDC APCs or tumor cell targets we analyze the T cells for CD137 upregulation by flow cytometry the following day. Reactive T cells, if any, are bulk sorted and expanded for re-screening downstream.

Results of T cell Tumor Reactivity Assays

In the past year, we focused on attempts to identify tumor reactive T cells. Our data showed that low percentages (<5%) of either tumor, PBMC, or TDLN T cells react to either cultured autologous tumor cells or mDCs loaded with BC cell line lysates (Figure 2 and 3). Surprisingly, these low percentages of reactivity remain unchanged even with the addition of PD-1 and PD-L1 blocking antibodies to these assays. This led us to attempt to examine reactivity in different ways. One such attempt involved adding IL-7 and IL-15 to single cell suspensions of digested tumor and examining T cells from this mixture the following day for CD137 expression [2]. On average, however, T cell reactivity remained below 5%. Another method involved extracting T cells from tumor fragments using high doses of IL-2 (2,000IU/ml) and reacting them with cryopreserved and thawed tumor fragments [3]. Again, even with blocking antibodies for PD-1, PD-L1, or TIGIT, reactivity remained below 5%.

Despite expecting higher levels of reactive T cells, we have also examined these patient samples T cells by high dimensional flow cytometry to try to understand their functional status better. The low reactivity data for these T cells supports the hypothesis that (1) these T cells are exhausted dysfunctional T cells, (2) these T cells lack tumor antigen specificity, or (3) cancer cells isolated from patient samples downregulated MHC I/target expression. We set out to understand the role(s) which each of these play. While we have found that the majority of TIL CD8+ T cells express high levels of PD-1 and TIGIT, they do not express other checkpoint molecules such as LAG-3 and TIM-3 (Figure 4). Furthermore, these cells express low levels of activation markers such as CD137 and OX-40. In this they differ from other cancer types, such as melanoma. Currently we are further assessing the functional status of TIL CD8+ T cells by assessing cytokine production, proliferation, and other surface molecules.

Enhancing MHC I expression on breast cancer cells

It has been well established that cancer cells are capable of evading the host immune response by downregulating MHC Class I and Class II molecules thus impairing T cell recognition to tumor associated antigens (TAA). To elicit an effective anti-tumor response for CD8 T cells, MHC I expression is required on cancer cells. Low reactivity T cell numbers from screening CD8 T cells with autologous tumor cells lead us to assess the levels of MHC I expression on patient tumor cells. Interferons (IFNs) have been shown to upregulate MHC I on cancer cells [4, 5]. However, IFNγ also induces PD-L1 expression on tumor cells [5]. We assessed the basal expression of MHC I and HLA-A2 on various breast cancer cell lines (Figure 5) which demonstrated variable expression of MHC I and HLA-A2. In addition, we measured the relative basal expression levels of MHC I on breast cancer patients CD326+ cancer cells via flow cytometry and immunohistochemistry (IHC) (Figure 6) and indeed MHC I expression of patient cancer cells are relatively low. We intend to further quantitate the *in vivo* basal expression of MHC I on patient tissues and compare to normal breast tissue and tonsil tissue (positive control) using IHC and analyzing with our inForm software. Because breast cancer patients cancer cells express low levels of MHC I, we investigated alternative methods to induce MHC I expression without increasing PD-L1 expression. Type I IFNs, histone deacetylase (HDAC) and MAPK inhibitors have been shown to upregulate MHC I expression [4] [6] [7]. We optimized conditions using breast cancer cell lines which were stimulated overnight with various concentrations of IFNα, IFNγ, and HDAC6 inhibitors and a single dose of a MAPK inhibitor. Some of the breast cancer cell lines were capable of inducing MHC class I expression by IFNα (BT474 and SKBR3) without upregulating PD-L1 expression (Figure 7). However PD-L1 expression levels on SKBR3 and MDA-MB-468 increased upon IFNγ stimulation. In addition, if MHC expression was already relatively high on cancer cells, they do not appear to upregulate MHC I further (MDA-MB-231). Histone modulators and the MAPK inhibitor did not significantly induce MHC I expression nor PD-L1 expression. Assessing the various concentrations of IFNs used in this study, we chose 750IU/ml of IFNα and 500IU/ml IFNγ for further testing on breast cancer patients tumor cells to induce MHC I expression.

To determine whether MHC I expression can be upregulated upon IFN stimulation in breast cancer patients cancer cells, we stimulated patients' tumor cells with 750IU/ml IFNα and/or 500IU/ml IFNγ overnight prior to co-incubating with autologous T cells for the reactivity assays. The next day, expression of MHC I and PD-L1 was assessed on tumor cells (CD326+) or non-tumor stromal cells (CD326-CD44+) using flow cytometry. Interestingly, MHC I and PDL1 expression were not induced in CD326+ cancer cells with either IFNγ or IFNα

stimulation (Figure 8). In contrast, non-tumor stromal cells (CD326-CD44+) significantly upregulated both MHC I and PD-L1 expression levels. We plan on interrogating both type I and type II IFN signaling pathways via activation of STAT1 (pSTAT1-Y701) and total STAT1 to determine if patient tumor cells are not capable of upregulating MHC I due to IFN signaling abnormalities in CD326+ cancer cells.

T cell killing of target cells

One of the read-outs to determine that CD8 T cells are indeed tumor reactive is to assess whether they kill tumor target cells. In order to assess CD8 T cell killing of autologous tumor cell targets, we use a colorimetric cytotoxicity assay which is a non-radioactive alternative to the ^{51}Cr release cytotoxicity assay. This assay indirectly measures lactate dehydrogenase (LDH) which is released upon cell lysis. To utilize this assay, the optimization of the effector and target cell numbers need to be determined to ensure an adequate signal-to-noise ratio. Per the manufacturer's suggestion, the best cell concentration to use as target cells are by generating absorbance values at least two times the background absorbance of the media control. For this experiment, we used a patients breast tumor cells (BC113), a breast cancer cell line (MCF7), and T cells from a healthy donor and ascertained their spontaneous release and maximum release of LDH. We determined that the minimum number of CD8 T cells to use in the cytotoxicity assay needs to be ~25,000 and that the minimum number of patient tumor cells or cells from breast cancer cell line needs to be ~5,000 cells. To test these cell numbers, we conducted a pilot study where we pulsed an HLA-A2+ breast cancer patients tumor target cells with 1ug/ml of pp65 peptide for one hour. A CMV specific T cell clone (499C.4) was used as the effector cells. We then co-cultured the cells together at a 10:1 and 5:1 Eff:Targ ratio (target cells at 10,000 each), or a 20:1, 10:1, and 2:1 Eff:Targ ratio (target cells at 5,000 each). The best ratio for yielding the best cytotoxicity was the 10:1 (10,000 target cells) and 20:1 (5,000 target cells) (28.4% and 24.8%, respectively). Going forward we will utilize similar Eff:Targ ratios and cell numbers for the cytotoxicity assays.

Peptide Screening

Peptides eluted from the surface of HLA-A2 breast cancer cell lines, provided by the OHSU team, were screened for specificity to naïve CD8 T cells isolated from the peripheral blood of healthy HLA-A2+ donors. By screening these peptides against healthy donors, we would be able to determine whether these peptides are immunogenic producing antigen-specific activation of naïve CD8 T cells. A total of 30 pools of 4-5 peptides per group were provided by the OHSU. In order to screen these peptides, we isolated naïve CD8 T cells and monocytes, which were differentiated into mDCs [8]. Once mDCs were matured with LPS and pulsed with 2µg/ml of total peptide. The following day these cells were co-cultured with naïve autologous CD8 T cells for 10 days in the presence of IL-2 and IL-15. Approximately one well of 0.5 million T cells is thought to be enough to identify antigen specific T cells with an approximate frequency of 0.1% within the naïve T cell repertoire of a healthy donor. On day 10, T-cells were collected and screened against the artificial APC cell line HMY-A2. The T cell activation markers CD137 and CD107 were assessed the following day by flow cytometry. 15 peptide groups were screened as all peptide groups displayed low percentages of CD107 mobilization and upregulation of CD137 (Figure 9).

Summary of Plans for Future Work

We had a recent project teams meeting in Denver and outlined an action list for each team to carry out over the next 6-12 months. The primary focus of the CoH team is to continue our search for tumor reactive T cells in BC patients and to sequence and get the top shared TCR sequences of CD8 T cells from the tumor, TDLN, and peripheral blood. These sequences will be used to make avatars and these avatars will be screened against HLA-A2+ BC cell lines, Oregon's eluted peptides, and Denver's Baculovirus library.

Outline of the project plan for the next 12 months

- Continue to screen patient T cells for reactivity against autologous tumor cells.
- Continue to send patient T cells to Denver for TCR repertoire analysis.
- Generate T cell clones via FACS sorting that are reactive to tumor antigen.
- Sequence TCR CDR3 region of identified tumor reactive T cells.
- Further assessing the functional status of TIL CD8+ T cells by assessing cytokine production, proliferation, and other surface molecules.
- Interrogating the IFN signaling pathways of patient tumor cells.

Personnel

1. Peter P. Lee, MD – project PI (40% effort)
2. John Yim, MD – CoH Surgical Oncology (5% effort)
3. Joanne Mortimer, MD – CoH Medical Oncology (no salary requested)
4. Tommy Tong, MD – CoH Pathology (no salary requested)
5. Sailesh Pillai, PhD – Assistant Research Professor (no salary requested)
6. Colt Egelston, PhD – post doc (100% effort)
7. Diana Simons – Research Associate II (95% effort)
8. Grace Jimenez – Research Associate I (100% effort)
9. Gilbert Acosta – Research Associate I (50% effort)

KEY RESEARCH ACCOMPLISHMENTS

- Isolated and sent CD8 and CD4 T cells from 17 patients and 26 samples to Denver for TCR repertoire analysis in 2015
- Screened 34 patients for tumor reactive T cells and sorted reactive T cells for downstream analysis in 2015
- Sequenced the TCRs of generated T cell clones
- Optimized effector and target cell numbers and ratios for T cell cytotoxicity assays.
- Identified that BC patient tumor-T cell reactivity remains unchanged by the addition of PD-1 and PD-L1 blocking antibodies.
- Discovered the majority of CD8 TILs express high levels of PD-1 and TIGIT but do not express other checkpoint molecules, such as LAG-3 and TIM-3, and express low levels of activation markers, CD137 and OX-40, respectively.

REPORTABLE OUTCOMES

None at this time, but we are in the process of preparing 2-3 manuscripts for submission soon.

CONCLUSION:

Over the last 12 months of this award, we have focused on the identification of tumor-reactive T cells. We have worked out cytotoxicity assays to screen BC patients' tumor specific CD8 T cells against autologous tumor cells. We have identified TCR sequences in BC81 CD8 T cell clones which downstream analysis of antigen specificity will be assessed by the Denver team. The identification of these T cells will lead to downstream antigen discovery.

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APPENDICES:
None at this time

SUPPORTING DATA:

Table 1. Patient Characteristics					
Code	**Prior Therapy**	**Diagnosis**	**ER/PR/Her2**	**Molecular Subtype**	**Stage**
BC107	N	IDC	+/+/-	Lum	IIB
BC113	N	IDC	+/+/-	Lum	IIA
BC118	N	IDC	+/+/-	Lum	IIA
BC124	Y	IDC	+/+/+	Lum	N/A
BC128	N	PHYLLODES	N/A	N/A	N/A
BC131	N	BRCA+	N/A	N/A	N/A
BC132	Y	IDC RECURRENT	+/+/-	Lum	IIIA
BC145	Y	IDC	+/-/eq	Lum	IV
BC150	Y	IDC	+/-/-	Lum	IV
BC151	Y	ADENOCARCINOMA	-/-/-	Basal	IV
BC152	Y	ADENOCARCINOMA	-/-/+	Her 2	IV
BC154	N	IDC	+/+/-	Lum	IA
BC155	N	ILC	+/+/-	Lum	IIB
BC157	N	IDC	-/-/-	Basal	IA
BC160	Y	IBC	-/-/+	Her 2	IV
BC164	N	IDC	+/+/-	Lum	IA
BC166	N	IDC	+/+/+	Lum	IIB

N/A: not available; eq: equivocal

Table 2. Samples sent for HLA typing		
Sample Label	**HLA-A Type**	**HLA-DRB1 Type**
BC113	01:01 02:01	03:01 11:01
BC118	02:01 24:02	04:07 14:06
BC124	02:01 03:01	04:03 16:01
BC132	02:01 24:02	7:01

Table 3. TCR sequences of clones for patients BC85 and BC81. Shaded clones contain the dominant sequence found in patient BC81.							
Sample	**CDR3β**	**TRBV**	**TRBJ**	**TRBD**	**CDR3α**	**TRAV**	**TRAJ**
BC85							
4	CASVGGLAGGPNSYEQYF	TRABV6-2/ TRABV6-3	TRABJ2-7	TRBD2	CAPLDDKIIF	TRAV1-2	TRAJ30
BC81							
48	CASSFYRFPSEQYF	TRBV27	TRBJ2-7	TRBD1	CAGEVRGAQKLVF	TRAV27	TRAJ54
5	CASSLHPTGNGYTF	TRBV27	TRBJ1	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAJ52
3	CASSSHSKPGTGVMGGYTF	TRBV7-3	TRBJ1-2	TRBD1	CAERPANNARLMF	TRAV5	TRAJ31
2	CASSLHPTGNGYTF	TRBV27	TRBJ1-2	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAJ52
1	CASAEWASPSYEQYF	TRBV6-1	TRBJ2-7	TRBD1	CAMRFQPSNSNSGYALNF	TRAV14/DV4	TRAVJ41
40	CASSLHPTGNGYTF	TRBV27	TRBJ1-2	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAJ52
43	CASSSERDTGELFF	TRBV5-1	TRBJ2-2	TRBD2	CALGSWGKLQF	TRAV6	TRAJ24
44	CASSIHPTGNGYTF	TRBV27	TRBVJ1-2	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAJ52
52	CGPNGKEGEQNF	TRBV7-8	TRBJ2-7	TRBD1	CAVSSFRSNDYKLSF	TRAV1-2	TRAJ20
58	CASSFYRFPSEQYF	TRBV27	TRBJ2-7	TRBD1	CAGEVRGAQKLVF	TRAJ54	TRAV27
12	CASSLHPTGNGYTF	TRBV27	TRBJ1-2	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAJ52
13	CASSLHPTGNGYTF	TRBV27	TRBJ1-2	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAJ52
17	CASAEWGSPSYEQYF	TRBV6-1	TRBJ2-7	TRBD1	CAETRNDYKLSF	TRAV5	TRAJ20
24	CASSLHPTGNGYTF	TRBV27	TRBJ1-2	TRBD1	CATDSHAGGTSYGKLTF	TRAV17	TRAV52
26	CATNVYGAGGELFF	TRBV2	TRBJ2-2	TRBD1	CAYIQGAQKLVF	TRAV27	TRAV54

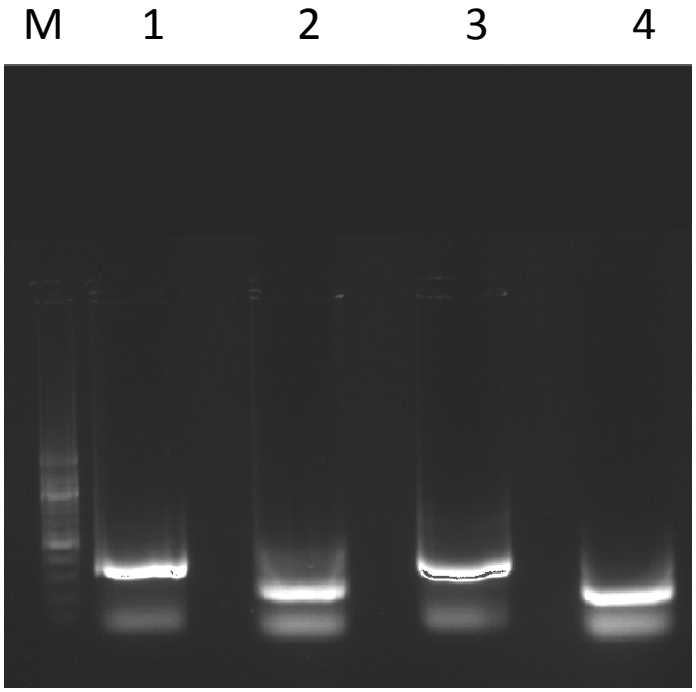

Figure 1. Electrophoresis of Vα and Vβ amplicons visualized after PCR reaction. cDNA from two different clones produce DNA fragments for Vα (Lane 1 &3) and Vβ (Lane 2 &4). The DNA fragments produced Vα at a length of 300bp and Vβ of 200bp, identified using a 100bp ladder (M).

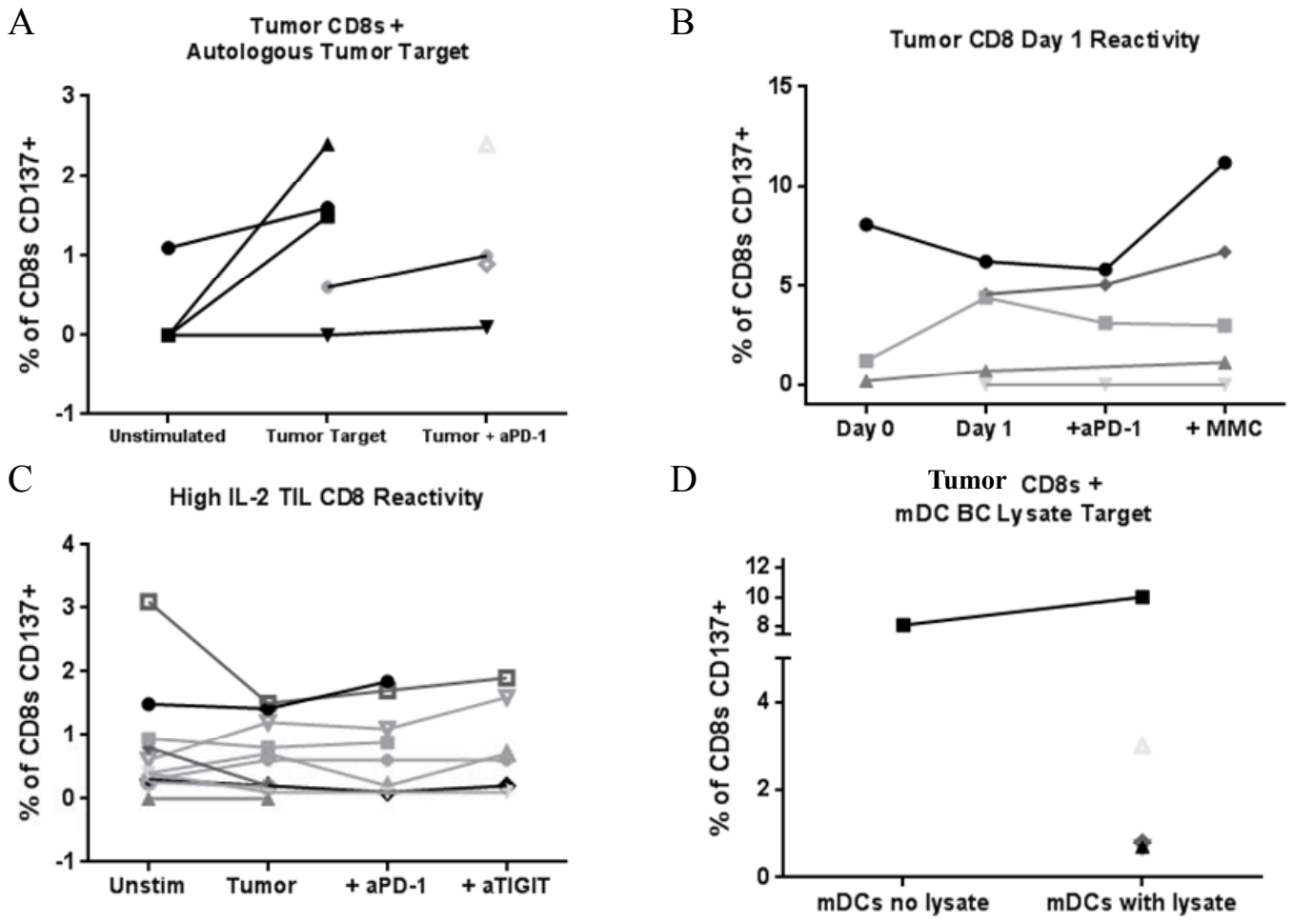

Figure 2. Reactivity of breast cancer patient tumor CD8+ T cells to tumor antigen. CD8+ T cells were isolated from patient tumors by either immediate digestion (A, D), by high IL-2 tumor fragment cultures (C), or left in the presence of autologous tumor cells overnight in combination with IL-7 and IL-15 (B). Isolated CD8+ T cells were then mixed with cultured autologous tumor cells (A), thawed cryopreserved tumor cells (C), or breast cancer cell line lysate loaded mDCs (D). Checkpoint molecule blocking antibodies (αPD-1/ αPD-L1 or αTIGIT) were included as shown. T cells were assayed for reactivity the following day by CD137 expression (data not shown).

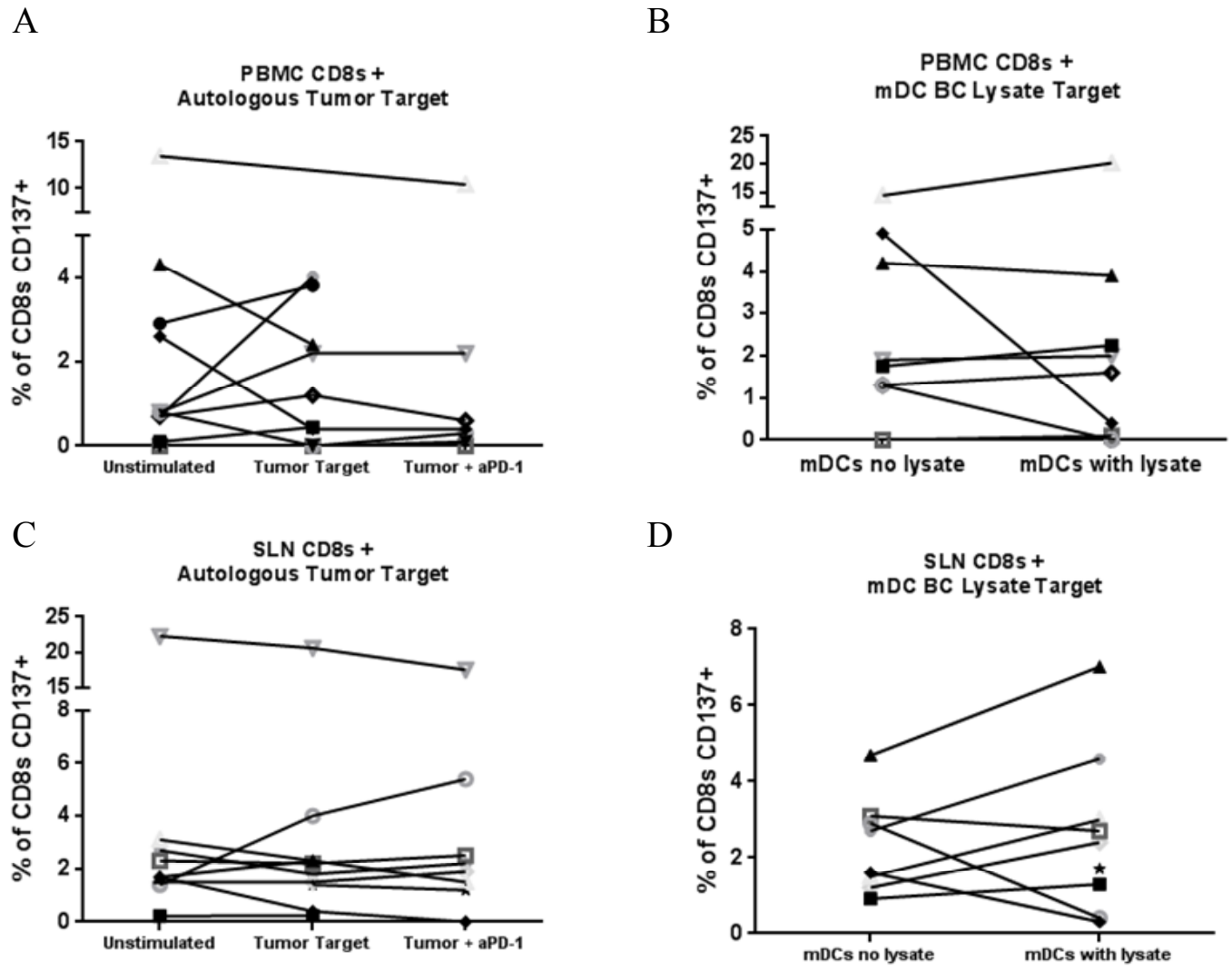

Figure 3. Reactivity of breast cancer patient PBMC and TDLN CD8+ T cells to tumor antigen. CD8+ T cells were isolated from patients PBMCs (A, B) or sentinel lymph nodes (SLN) (C, D). Isolated CD8+ T cells were then mixed with cultured autologous tumor cells (A, C), or breast cancer cell line lysate loaded mDCs (B, D). Checkpoint molecule blocking antibodies (αPD-1/ αPD-L1) are included as shown. T cells were assayed for reactivity the following day by CD137 expression.

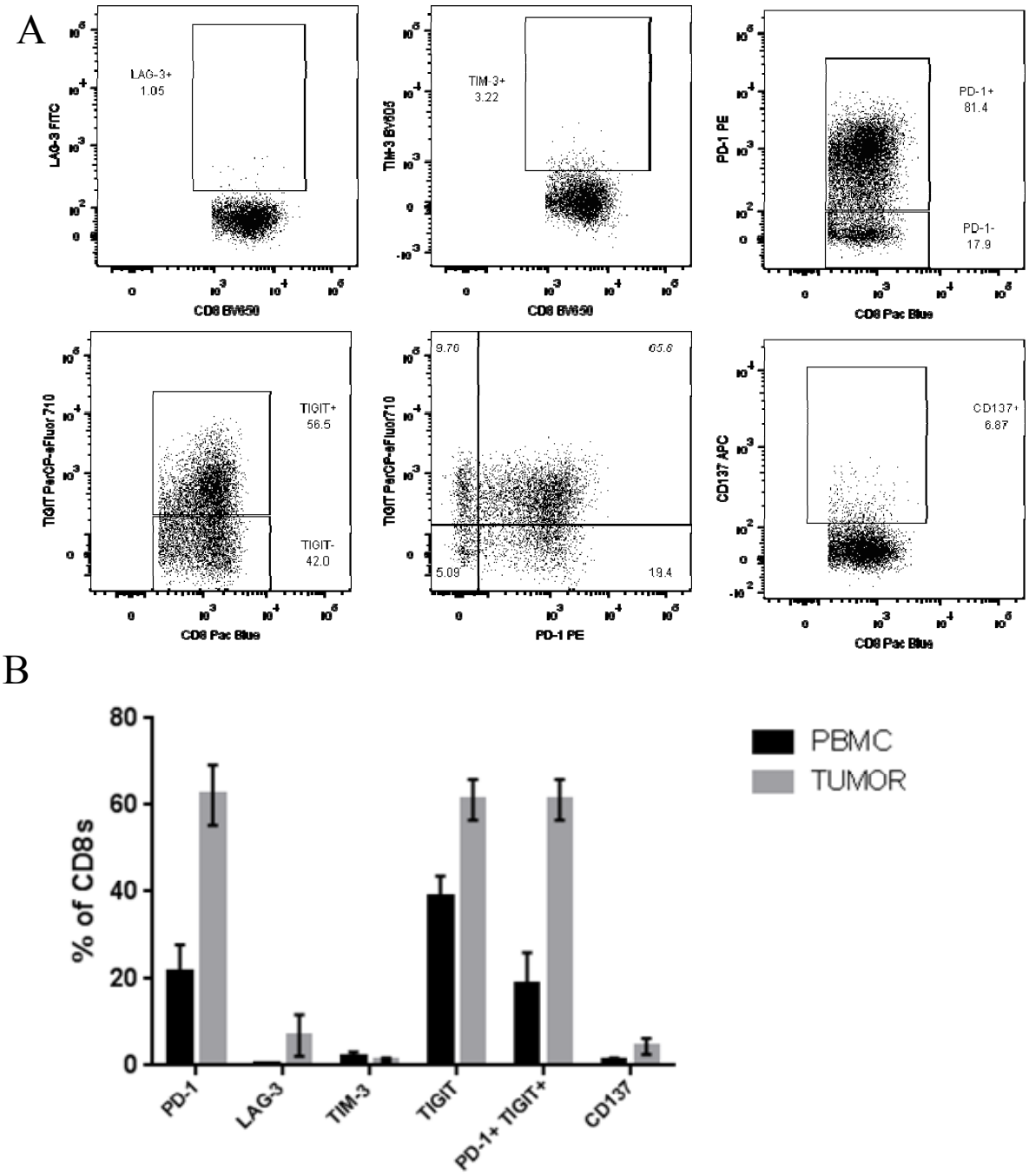

Figure 4. TIL CD8 expression of checkpoint and costimulatory molecules. Freshly isolated TILs or PBMCs were assayed by flow cytometry for surface expression of PD-1, LAG-3, TIM-3, TIGIT, and CD137 (4-1BB). Summary data (B) and representative data (A) are shown.

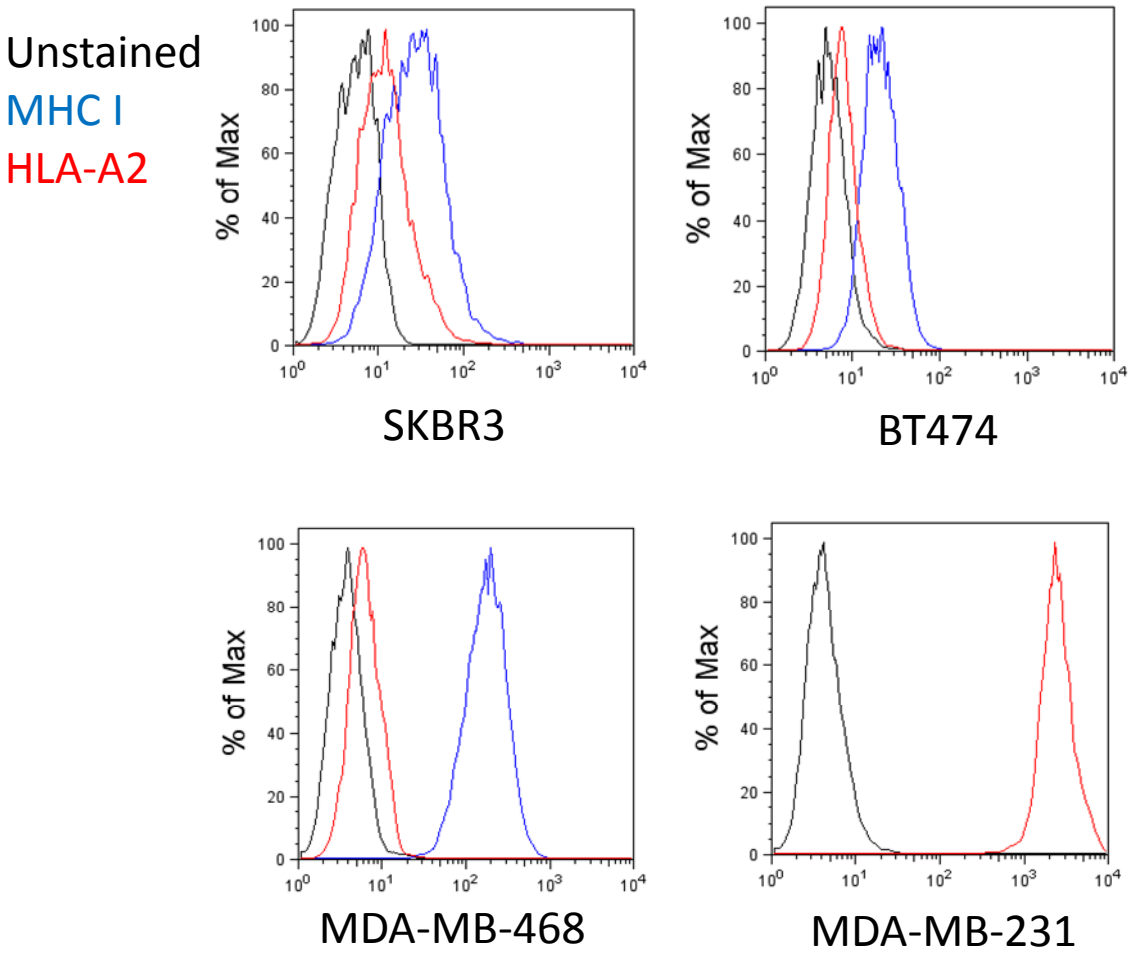

Figure 5. MHC I and HLA-A2 expression on breast cancer cell lines. Breast cancer cell lines were stained to measure the expression levels of MHC I (blue), HLA-A2 (red), or remained unstained (black) using flow cytometry.

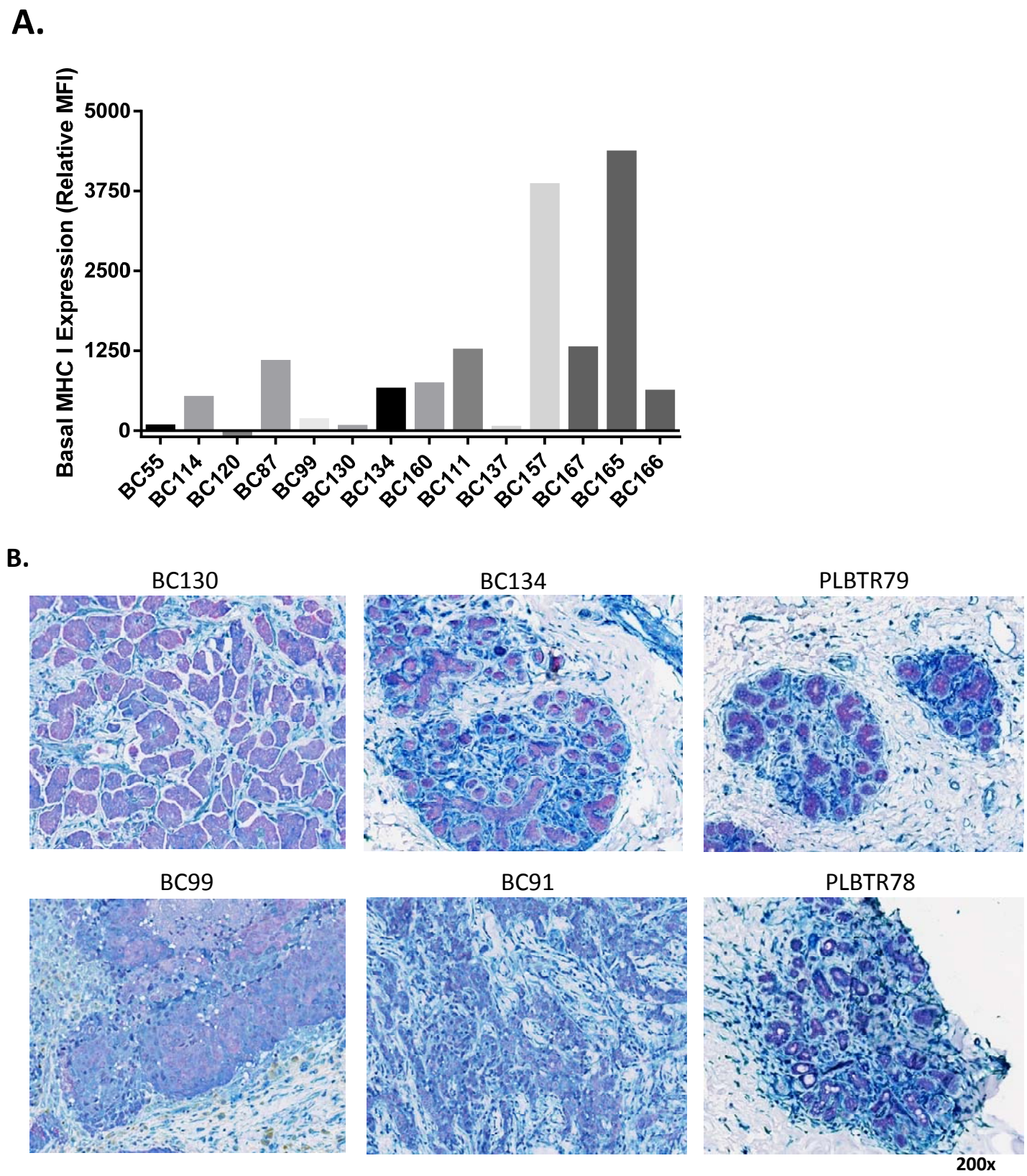

Figure 6. Basal expression of MHC class I on breast cancer patients' tumor cells. (A) Relative MHC I basal expression levels were calculated based on the median fluorescent intensity (MFI) of MHC I expression minus the median MFI of a matched isotype control. (B) Breast cancer patients paraffin-embedded tumor tissue was stained with MHC I (dark blue), pan-cytokeratin (pink), and counter stained with hematoxylin. Co-expression of cytokeratin and MHC I can be visualized by a gradient from light purple to darker purple depending on the levels of MHC I expression.

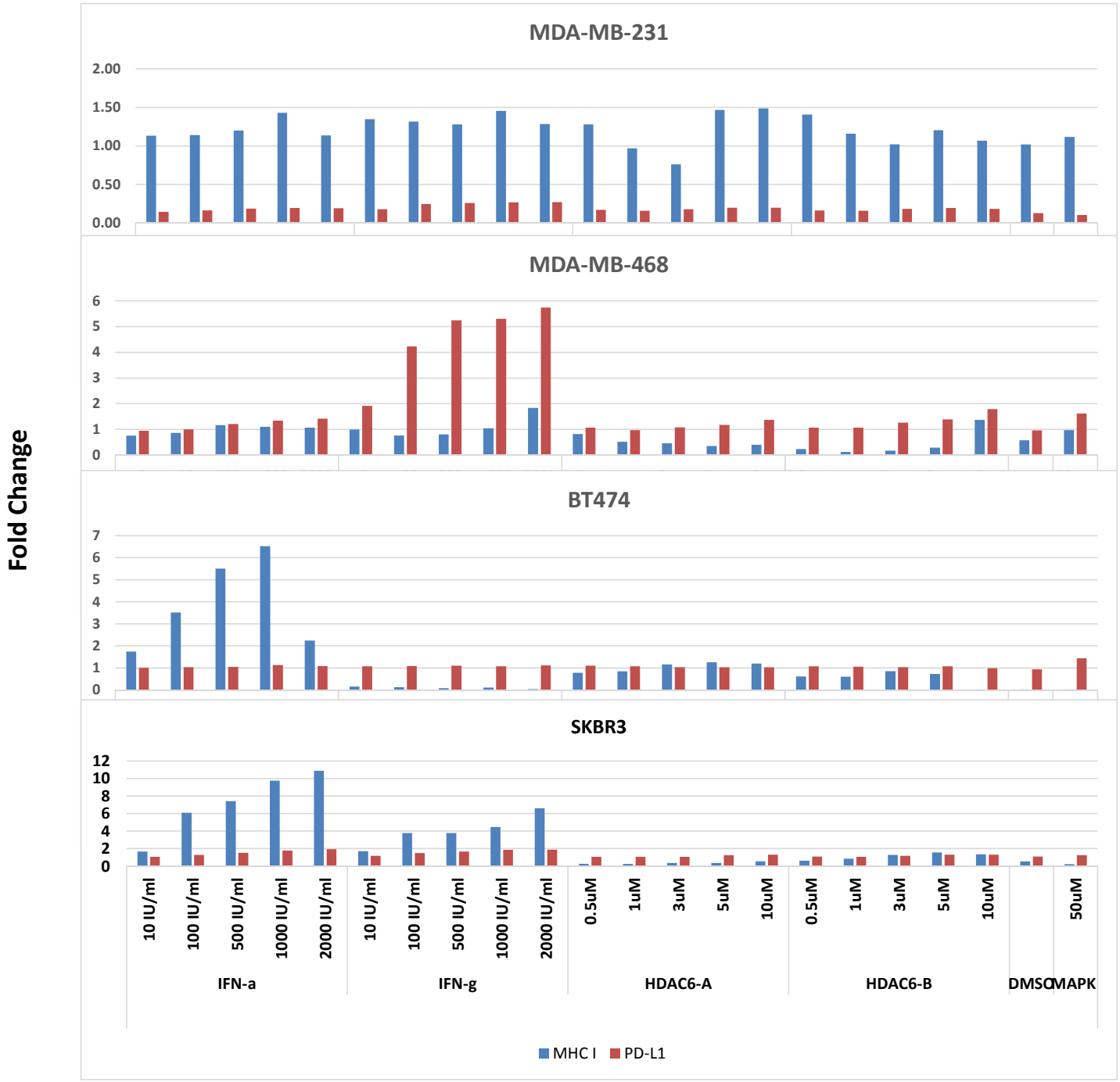

Figure 7. MHC I expression on breast cancer cell lines induced by IFNs, HDAC inhibitors, and MAPK inhibitors. Breast cancer cell lines were stimulated with various concentrations of IFNα, IFNγ, histone deacetylase inhibitors (HDAC6A/B), or a MAPK inhibitor, or remained unstimulated and the induction of MHC I (blue) and PD-L1 (red) expression was assessed. The fold change is the median stimulated MFI divided by the median MFI of the unstimulated cells. DMSO was used as a control for both the HDAC6A/B and MAPK inhibitors.

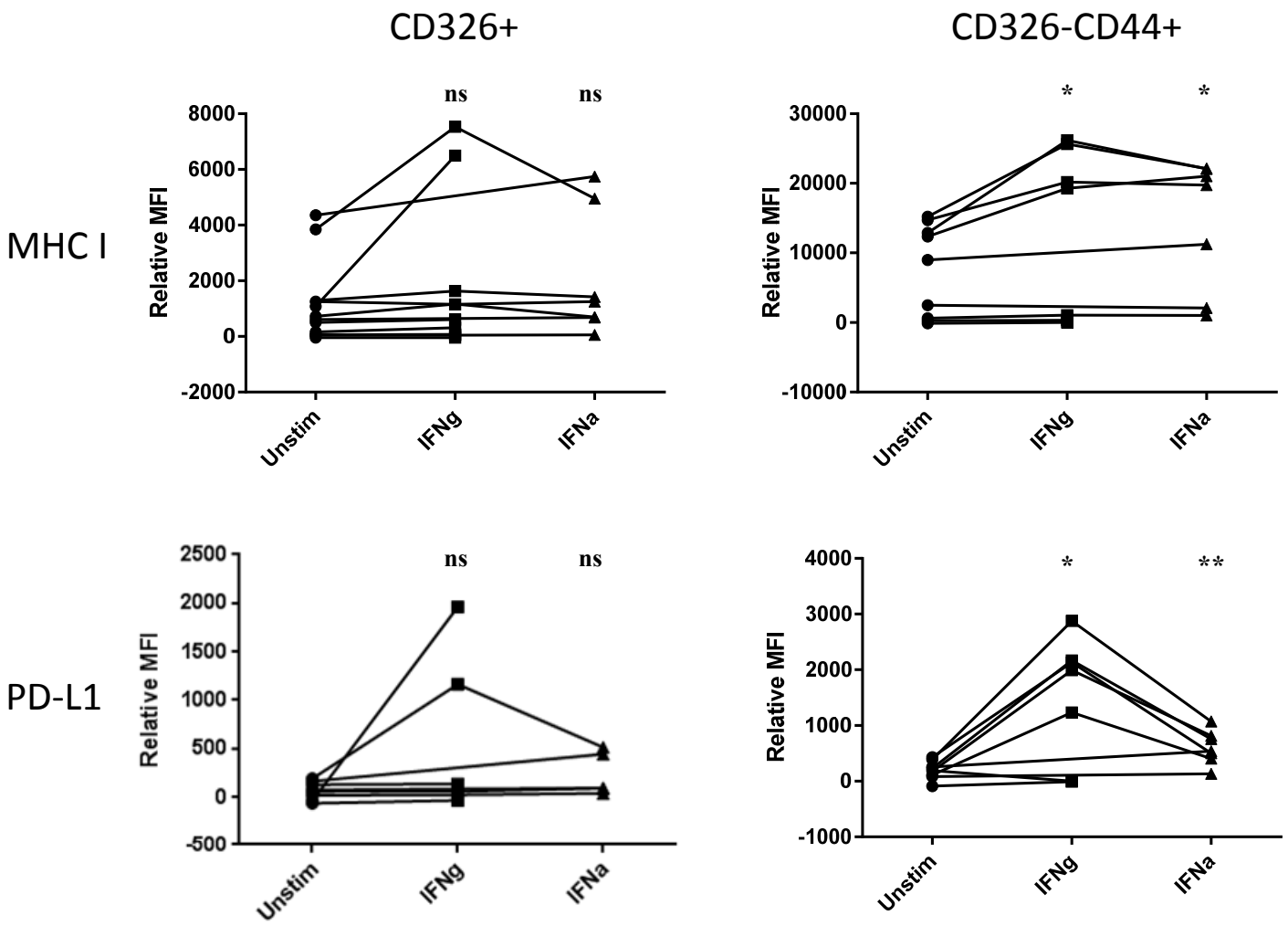

Figure 8. Induction of MHC I and PD-L1 expression by interferons (IFN) on breast cancer patients tumor cells and non-tumor stromal cells. Breast cancer patients CD326+ tumor cells and CD326-CD44+ non-tumor stromal cells were stimulated overnight with 500IU/ml IFNγ, 750IU/ml IFNα, or remained unstimulated. The relative MFI was measured by subtracting the unstimulated or stimulated median MFI from the median MFI of a matched isotype control. Ns= not significant. (*) = $p<0.05$; (**) = $p<0.01$

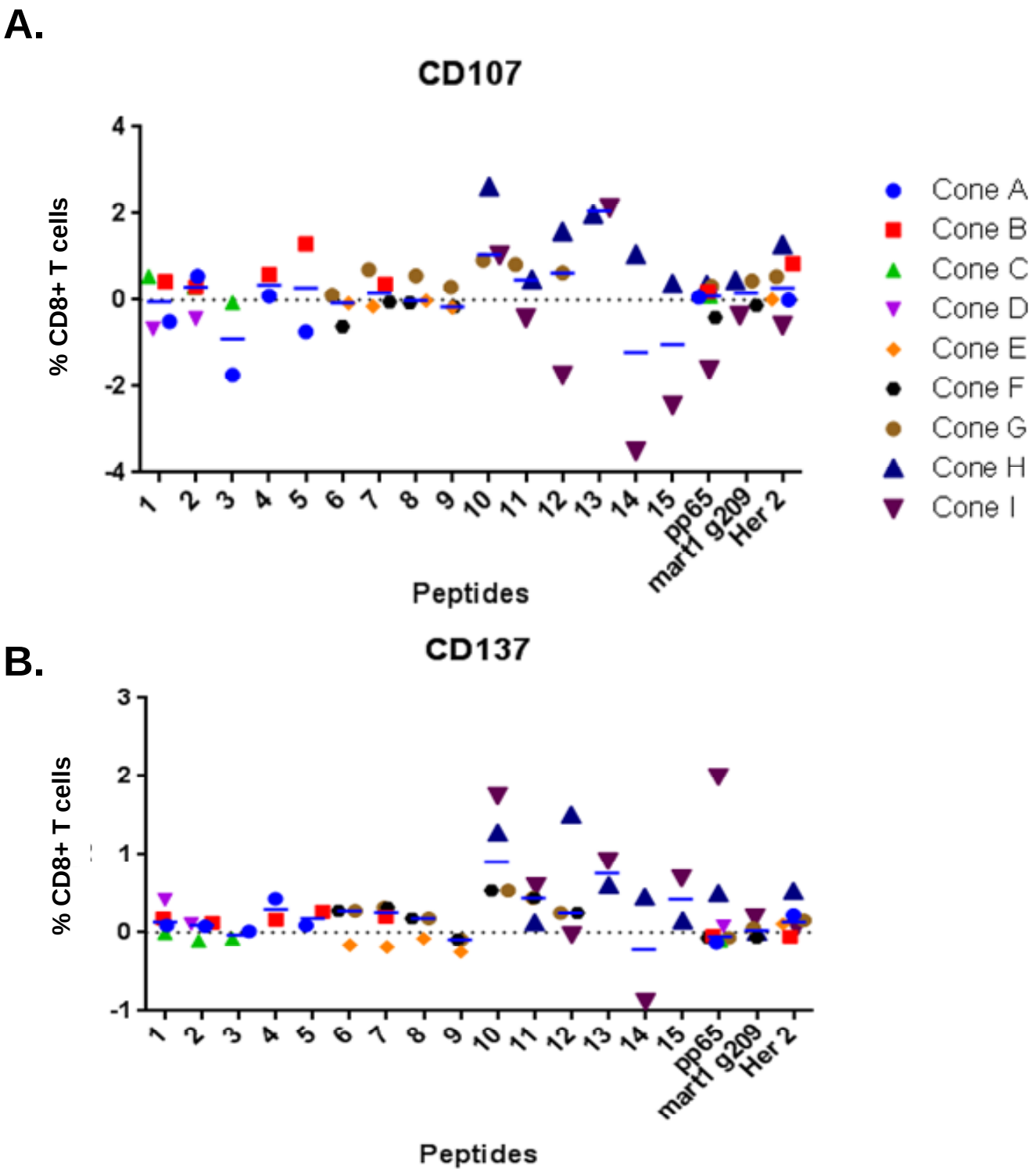

Figure 9. Percentage of CD107 mobilization and upregulation of CD137when screened against breast cancer cell lines peptides. Peptide lysate pools 1-15 were generated from HLA-A2+ breast cancer cell lines and were screened against healthy donors naïve CD8+ T cells isolated from peripheral blood where (A) CD107 mobilization and (B) CD137 activations was assessed after 4 hours or overnight, respectively. pp65 and Mart1 g209 peptides were used as positive controls.